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Intrinsic bacterial biodegradation of petroleum contamination demonstrated *in situ* using natural abundance, molecular-level ^{14}C analysis

Gregory F. Slater [a,*], Robert K. Nelson [b], Brian M. Kile [b], Christopher M. Reddy [b]

[a] *School of Geography and Geology, McMaster University, 1280 Main Street West, Hamilton, Ont., Canada L8S 4K1*
[b] *Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA*

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Abstract

Natural abundance, molecular-level ^{14}C analysis was combined with comprehensive gas chromatography (GC × GC) to investigate, *in situ*, the role of intrinsic biodegradation in the loss of petroleum hydrocarbons from the rocky, inter-tidal zone impacted by the *Bouchard 120* oil spill. GC × GC analysis indicated accelerated losses of *n*-alkane components of the residual petroleum hydrocarbons between day 40 and day 50 after the spill. ^{14}C analysis of bacterial phospholipid fatty acids (PLFA) from the impacted zone on day 44 showed that the polyunsaturated fatty acids attributed to the photoautotrophic component of the microbial community had the same $\Delta^{14}C$ as the local dissolved inorganic carbon (DIC), indicating that this DIC was their carbon source. In contrast there was significant $\Delta^{14}C$ depletion in the saturated and mono-unsaturated PLFA indicating incorporation of petroleum carbon. This correlation between the observed accelerated *n*-alkane losses and microbial incorporation of $\Delta^{14}C$-depleted carbon directly demonstrated, *in situ*, that intrinsic biodegradation was affecting the petroleum. Since the majority of organic contaminants originate from petroleum feed-stocks, *in situ* molecular-level ^{14}C analysis of microbial PLFA can provide insights into the occurrence and pathways of biodegradation of a wide range of organic contaminants.

1. Introduction

Microbial communities play significant roles in systems ranging from carbon cycling over global and geologic timescales, to microbial transformation of organic contaminants in the modern environment. While genetic approaches have made substantial progress in characterizing the constituents of environmental microbial communities (Keller and Zengler, 2004; Tringe et al., 2005; Walker et al., 2005), *in situ* characterization of the functional activity of the latter continues to be challenging (Tyson et al., 2004). Demonstrating microbial metabolism of organic contaminants is particularly important in assessing the role of intrinsic bioremediation in degrading, and therefore mitigating the impact of, organic contamination resulting from industrial and human activities. Accurate assessment of the role of microbial communities in degradation is essential for effective environmental risk

* Corresponding author. Tel.: +1 905 525 9140x26388; fax: +1 905 546 0463.
E-mail address: gslater@mcmaster.ca (G.F. Slater).

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assessment and remediation efforts. However, in complex natural systems it is often difficult to determine, *in situ,* the occurrence or significance of intrinsic biodegradation processes, particularly where multiple mechanisms of mass loss may occur. This is of particular importance as shipping activities, and exploration and exploitation of petroleum, increase in remote regions (i.e., the arctic) where remediation and restoration efforts may be hampered by difficulty in accessing or processing contaminated materials.

A wide range of approaches are currently applied to investigate and monitor intrinsic biodegradation in the environment. These provide essential understanding of the process, but in some cases they do not have sufficient resolution to differentiate between degradative and non-degradative processes of mass loss, or between metabolism of natural and contaminant organic compounds. Techniques that rely on accumulated evidence, such as changes in contaminant compound distribution (Volkman et al., 1984; Mills et al., 2003) may record losses due to both degradative and non-degradative processes. Microbial culturing and genetic analysis of microbial communities can identify the presence of organisms in an environmental sample, but not that a particular metabolic reaction is actively occurring *in situ*. Compound-specific stable isotopic analysis of reactants and products can overcome some challenges faced by analysis of concentrations alone. However, due to variations in isotopic fractionation and signal dilution as the number of carbon atoms present in a molecule increases (Slater, 2003; Schmidt et al., 2004; Sun et al., 2004) this approach is not successful in many situations. Natural abundance variations in $\delta^{13}C$ of microbial lipids can differentiate carbon sources and metabolic activities of microbes producing certain biomarker compounds (Boschker and Middleburg, 2002). Unfortunately, in many systems insufficient isotopic distinction between ''natural'' and ''contaminant'' carbon sources exists, and the effects of variability in isotopic fractionation by biosynthetic pathways (Hayes, 2001) cannot be well constrained. Isotopic tracers have been used in many studies to elucidate microbial metabolism using ^{14}C- and ^{13}C-labeled substrates and their incorporation into microbial cells (Hesselsoe et al., 2005), lipids (Roslev et al., 1998), and nucleic acids via stable isotope probing (Radajewski et al., 2000). However, addition of labeled substrates can be costly, difficult to constrain, involve health concerns and have higher bioavailability than contaminants present for long time periods; i.e., Steinberg et al. (1987) observed that ^{14}C-labeled ethylene dibromide (EDB) added to EDB-contaminated soils was metabolized preferentially over the native contaminant. In cases where organic contaminants originate from petroleum carbon, natural abundance molecular ^{14}C analysis represents a new approach that can overcome these challenges and directly identify and assess, *in situ*, microbial metabolism and cycling of carbon in the environment.

Biodegradation of petroleum hydrocarbons and petroleum-based synthetic compounds is an optimal system for this approach due to the geologic age of petroleum-derived carbon, which contains no detectable ^{14}C ($\Delta^{14}C = -1000‰$). In contrast, recently photosynthesized organic carbon contains modern levels of ^{14}C ($\Delta^{14}C = 100 \pm 50‰$ depending where and when carbon fixation occurred). Further, any isotopic fractionation during biosynthesis or other processes is normalized to $\delta^{13}C = -25‰$ during $\Delta^{14}C$ analysis, removing any variation associated with these factors. Characterizing the $\Delta^{14}C$ content of a biomolecule can thus be used to assess the relative contribution of petroleum versus modern carbon during its synthesis. This approach has been used to: demonstrate a natural source for a bioaccumulating methoxylated polybrominated diphenyl in a North Atlantic True's beaked whale (Teuten et al., 2005); identify CO_2 fixation of ^{14}C-depleted deep water CO_2 by archea in the modern ocean (Pearson et al., 2001); demonstrate incorporation of fossil carbon in fauna living around a hydrocarbon seep (Bauer et al., 1990); and highlight microbial assimilation of shale organic carbon into phospholipid fatty acids (PLFA) in a laboratory enrichment culture (Petsch et al., 2001). Recently, this approach has also been used to investigate microbial carbon sources in contaminated salt marsh sediments. Slater et al. (2005) used this approach to demonstrate that there was no significant microbial metabolism of petroleum hydrocarbons persisting at depth in salt marsh sediments. In contrast, Wakeham et al. (2006) demonstrated a small amount of bacterial metabolism of ^{14}C-depleted carbon, likely derived from petroleum contaminating surface salt marsh sediments. However, no study to date has directly related petroleum hydrocarbon mass losses with microbial incorporation of ^{14}C-depleted carbon and therefore demonstrated that molecular-level ^{14}C analysis can provide *in situ* demonstration of biodegradation

with temporal resolution relevant to environmental remediation.

Microbial PLFA represent an ideal substrate to isotopically investigate intrinsic remediation via microbial biodegradation. Phospholipids are integral components of cell membranes that are labile and hydrolyze within weeks after cell death (White et al., 1979; Harvey et al., 1986). Consequently, the isotopic composition of *in situ* microbial PLFAs reveals a near-instantaneous snapshot of the carbon source of the active microbial community at a site. Further, while humic acids co-extract and can confound natural abundance isotopic analysis of nucleic acids (Pelz et al., 1998), PLFA are amenable to gas chromatographic separation and can be collected in sufficient quantities from a site for natural abundance isotopic analysis. Natural abundance variations in ^{13}C of microbial lipids and incorporation of added tracers have been applied successfully in many studies. However, as discussed previously, in some cases they cannot provide *in situ* resolution of carbon sources and cycling. In such cases, natural abundance ^{14}C analysis of PLFA can provide a new approach for direct, *in situ* resolution of microbial uptake of contaminant organic carbon sources with $\Delta^{14}C$ distinct from the concurrent natural organic matter.

We have applied this approach to investigate the intrinsic biodegradation of petroleum hydrocarbons deposited on a rocky intertidal zone at Nyes Neck, MA following the *Bouchard 120* oil spill on April 27, 2003. The *Bouchard 120* spilled ~400,000 l of No. 6 (or Bunker C) fuel oil along the coastlines of Buzzards Bay, MA (Nelson et al., 2006). While this spill was the subject of extensive remediation efforts, it also provided an ideal opportunity to investigate the role of intrinsic biodegradation. When comprehensive GC × GC analysis indicated the increased occurrence of biodegradation of the *n*-alkane component of the petroleum hydrocarbons between day 40 and day 50 after the spill, we applied molecular ^{14}C analysis to demonstrate that this accelerated mass loss was directly related to biodegradation and utilization of the petroleum carbon.

2. Methods

2.1. Sample extraction and preparation

The distribution of petroleum hydrocarbons extracted from intertidal rocks at Nyes Neck shortly after the spill occurred was characterized by comprehensive two-dimensional gas chromatography (GC × GC). This novel technology affords much higher chromatographic resolution than traditional gas chromatography and can reveal the most subtle changes in hydrocarbon mixtures (Reddy et al., 2002). The extraction and analysis by GC × GC of oil-covered rocks from Nyes Neck is discussed elsewhere (Nelson et al., 2006). Briefly, the rocks were spiked with 168 μg of dodecahydrotriphenylene (internal standard) and then sonicated for 15 min in precombusted, glass jars containing 40 ml of a 90/10 mixture of dichloromethane (DCM)/methanol. The DCM extracts were dried over activated sodium sulfate and purified using fully-activated silica gel to isolate a combined saturate and aromatic fraction for GC × GC analysis.

Samples of the microbial community for PLFA distribution and isotopic (^{13}C, ^{14}C) analysis were collected on June 10, 2003 (day 44 after the spill) when the rate of *n*-alkane loss was maximal. The microbial community at the site exists as a thin film strongly adhered to the rocks. Microbial PLFA were extracted directly from the rocks by a modified Bligh and Dyer extraction and microbial phospholipids were purified via silica gel chromatography, and hydrolyzed to form fatty acid methyl esters (FAMEs) by mild alkaline hydrolysis (White and Ringelberg, 1998). Extraction of PLFA involved an initial 15 min sonication of the samples in a 2:1:0.8 methanol:chloroform:phosphate buffer solution followed by a quiescent 24-h extraction. Phospholipids were separated on a glass column packed with 10 g of silica gel (60 A, 100–200 mesh) into three fractions using 50 ml of $CHCl_3$ (f_1), 50 ml of acetone (f_2), and 200 ml of methanol (f_3). The microbial phospholipids were recovered in the f_3 fraction and were then hydrolyzed under alkaline conditions to produce fatty acid methyl esters (FAMEs) of phospholipid fatty acids (PLFA). The methanol used for methanolysis was characterized for its ^{13}C and ^{14}C content before the reaction so the contributions of the methanol-derived methyl groups to the FAMEs could be isotopically corrected.

The analysis of the FAMEs generated from the microbial phospholipids is described below. FAMEs were identified and quantified using gas chromatography–mass spectrometry (GC–MS), $\delta^{13}C$ analysis of the FAMEs was by gas chromatography-isotope ratio mass spectrometry (GC–IRMS) and collection and purification of FAMEs for ^{14}C analysis was by preparative capillary gas chromatography (PCGC) (Eglinton et al., 1996; Slater et al., 2005).

2.2. GC × GC analysis

The GC × GC system employed an Agilent 6890 gas chromatograph configured with a 7683 series split/splitless auto-injector, two capillary gas chromatography columns, a model KT-CLM-ZOE02 loop jet modulator (Zoex Corporation, Lincoln, NE), and a flame ionization detector. Columns were a non-polar 100% dimethyl polysiloxane phase (Restek Rtx-1 Crossbond, 7 m length, 0.10 mm i.d., 0.4 μm film thickness) followed by a 50% phenyl polysilphenylene-siloxane column (SGE BPX50, 0.82 m length, 0.10 mm i.d., 0.1 μm film thickness).

2.3. GC–MS analysis

A small fraction of the PLFA FAMEs mixture was analyzed for identification and quantification by gas chromatography–mass spectrometry on an Agilent 6890 gas chromatograph equipped with a J&W DB-XLB column (60 m; 0.25 mm i.d.; 0.25 μm film) and coupled to an Agilent 5973 MSD operating under electron ionization (EI, 70 eV) in the full scan mode. The oven temperature was initially held at 40 °C (1 min), then ramped at 20 °C min^{-1} to 130 °C, at 4 °C min^{-1} to 160 °C, and finally at 8 °C min^{-1} to 300 °C. Identification was based on mass fragmentation patterns and retention time as compared with a bacterial reference standard (Bacterial Acid Methyl Esters CP Mix, Matreya Inc.). PLFAs are designated by the number of carbon atoms and the number of double bonds.

2.4. GC–IRMS analysis

Compound-specific stable carbon isotope analysis of PLFA was performed in triplicate on a gas chromatography–isotope ratio mass spectrometry (GC–IRMS) system consisting of a Agilent 6890 gas chromatograph interfaced to a Finnigan-MAT DeltaPlus via a GC Combustion III interface. The GC was equipped with a CP-Sil 5CB column (50 m; 0.25 mm i.d.; 0.25 μm film). Samples were injected into a Gerstel CIS (PTV) injector initially at 10 °C (0.25 min hold) followed by a ramp at 12 °C s^{-1} to 350 °C (3 min hold); GC temperature program was initially held at 60 °C (3 min), then ramped at 20 °C min^{-1} to 130 °C, at 3 °C min^{-1} up to 260 °C and finally at 10 °C min^{-1} up to 340 °C and held for 2 min.

2.5. ^{14}C analysis

The isolation and analysis of the ^{14}C content of microbial FAMEs is described elsewhere (Slater et al., 2005). Briefly, concentrated solutions of PLFAs were repeatedly injected ~100 times onto a Hewlett–Packard 5890 gas chromatograph coupled to a Gerstel preparative fraction collector. The PLFAs were separated on a J&W DB-XLB column (60 m; 0.53 mm i.d.; 0.5 μm film) with hydrogen carrier gas. The oven temperature program was held for 1 min at 50 °C and ramped at 10 °C min^{-1} to 150 °C. The temperature was then ramped at 1.5 °C min^{-1} to 180 °C, held for 10 min, and then 10 °C min^{-1} to 320 °C. Individual PLFA were trapped in cooled (0 °C), glass u-tubes. Purified individual or groups of compounds were recovered with DCM and transferred to pre-combusted quartz tubes (12 mm × 20 cm). The solvent was evaporated under a stream of nitrogen and then ~100 mg of copper oxide was added to each tube. The tubes were evacuated on a vacuum line, sealed, and combusted at 850 °C for 5 h, yielding carbon dioxide, water, and other combustion gases. The tubes were reattached to the vacuum line and the carbon dioxide was isolated and purified through a series of cold traps and quantified by manometry. About 10% of the carbon dioxide was reserved for $\delta^{13}C$ analysis by isotope ratio mass spectrometry. The remaining 90% was reduced to graphite (McNichol et al., 1994). Targets of the graphite were pressed and mounted on target wheels for ^{14}C analysis by AMS at the national ocean sciences accelerator mass spectrometry (NOSAMS) facility at Woods Hole Oceanographic Institution, Woods Hole, MA (McNichol et al., 1994). In this study, all ^{14}C measurements are normalized to $\delta^{13}C$ values of −25‰ and expressed as $\Delta^{14}C$ values. The latter term is the per mil (‰) deviation from the international standard for ^{14}C dating, Standard Reference Material 4990B "*Oxalic Acid*" (McNichol et al., 1994). In this context, petroleum has a $\Delta^{14}C$ of −1000‰ while recently photosynthesized materials are more enriched (100 ± 50‰) depending on when and where the organism grew.

3. Results and discussion

3.1. Petroleum hydrocarbon distribution

During the first few weeks after the spill, we observed significant losses of lower molecular

weight hydrocarbons that have boiling points less than n-C_{20} alkane, resulting from evaporation and perhaps water washing. In addition, larger compounds also began to disappear shortly after the spill. Close inspection of the GC × GC images revealed that these losses were mainly limited to n-alkanes (Fig. 1). To compare the relative losses of these compounds in the spilled oil, we calculated the ratio of the n-alkanes to that of 17α(H),21β(H)-hopane, which is a biomarker present in most crude oils and in the *Bouchard 120* cargo. The latter compound resists biodegradation on short term time scales relevant to this study (Prince et al., 1994) and is not likely to biodegrade, evaporate, water wash, or degrade due to direct photolysis. Shown in Fig. 2 is a typical result of this

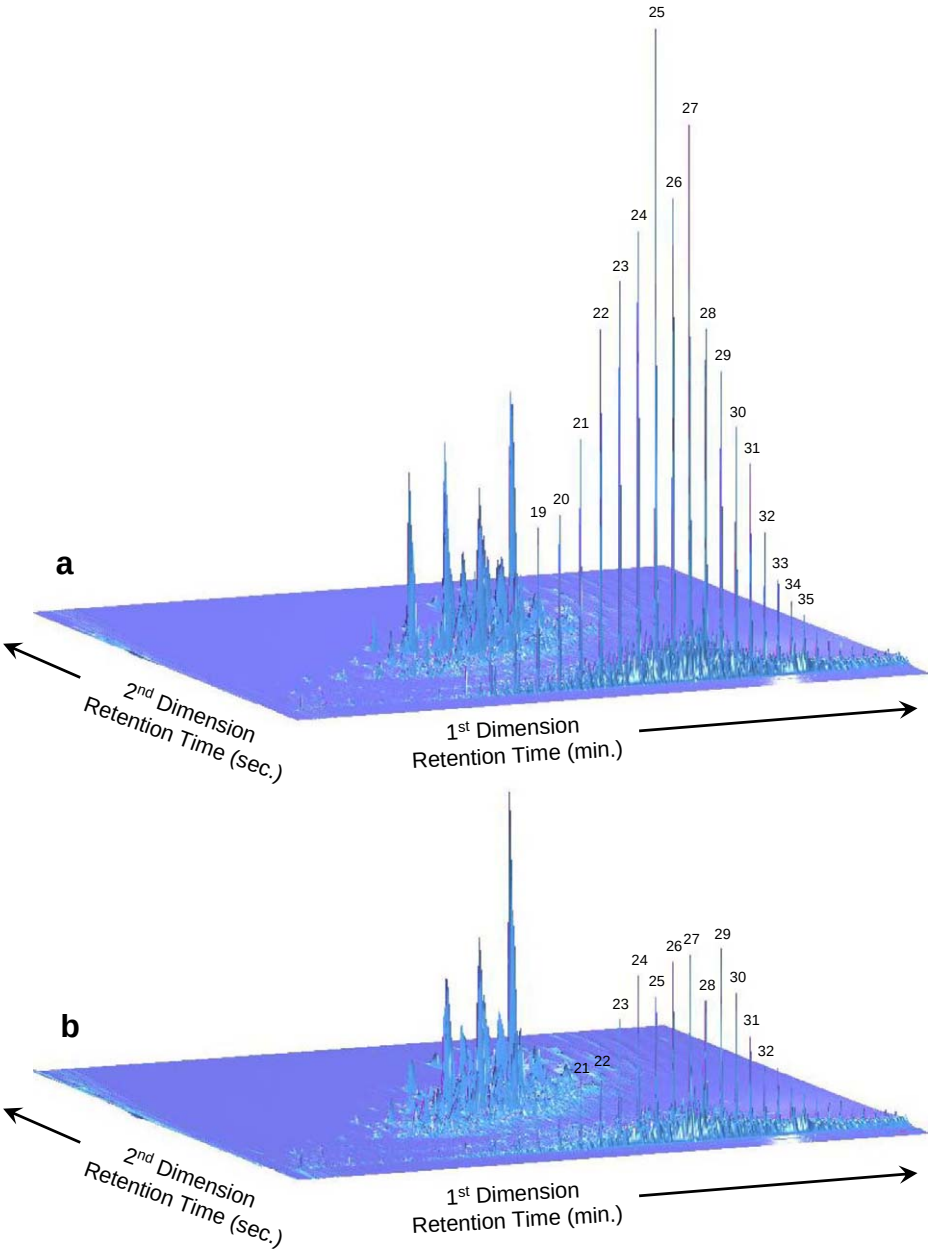

Fig. 1. GC × GC chromatograms of the petroleum hydrocarbons at the site on day 40 (a) and day 50 (b) after the spill illustrating the extent of loss of the n-alkane envelope (n-alkanes are denoted by carbon number at the front of the figures) relative to the aromatic components of the petroleum hydrocarbons that appear in the central region of the figures.

calculation with the ratio of n-C_{24} to 17α(H), 21β(H)-hopane over the first 200 days after the spill. It is clear that within 2 weeks, n-C_{24} quickly began to be removed from the bulk oil relative to 17α(H),21β(H)-hopane. However, on or about June 6 (day 40), there was a substantial increase in the loss of n-C_{24} and the other n-alkanes that continued until June 10 (day 50) (Fig. 1). We believe that this is due to biodegradation.

3.2. Microbial phospholipids

The distribution of the PLFA generated from microbial phospholipids extracted from the rocks at this site is shown in Table 1 and Fig. 3. The

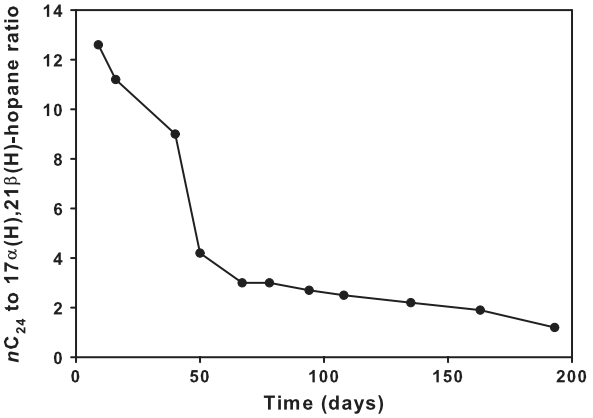

Fig. 2. Ratio of the n-C_{24} to 17α(H),21β(H)-hopane over the first 200 days after the spill. There is a significant increase in the rate of loss of n-C_{24} and other n-alkanes between day 40 and day 50 that accounts for 45% of the total losses over the first 200 days.

Table 1
The distribution (in mol%), $\delta^{13}C$ (in ‰) and standard deviation (1σ, $n = 3$) of PLFA in the microbial community sampled from Nyes Neck

PLFA	Mol%	$\delta^{13}C$ (‰)	Standard deviation
$C_{16:1}$	0.8	−22.1	2.1
$C_{16:0}$	18.9	−21.5	0.6
$C_{17:0}$	1.2	−22.1	0.2
$C_{18:2}$	1.1	−18.4	0.2
$C_{18:1}$	3.9	−16.9	0.1
$C_{18:1}$	4.4	−18.8	0.1
$C_{18:1}$	1.2	−21.7	0.2
$C_{18:0}$	15.2	−18.8	0.1
$C_{20\ unsat}$	46.9	−19.2	0.5
$C_{20:0}$	2.5	−22.5	0.1

95% confidence intervals (2σ) were thus 0.2–4.1‰, in general consistent with the ±0.5‰ accuracy and precision expected for GC–C–IRMS analysis.

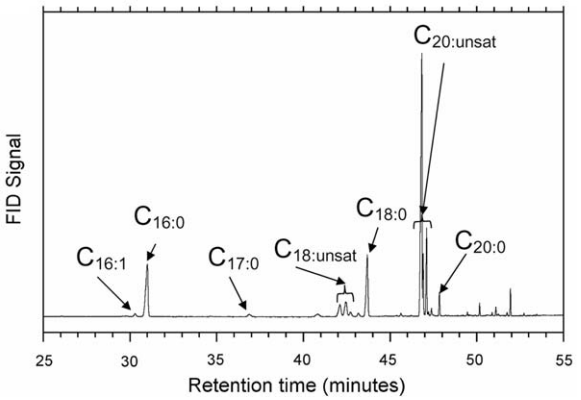

Fig. 3. PLFA distribution as observed by FID during PCGC collection of PLFA for molecular ^{14}C analysis, the PLFA collected for analysis are annotated.

majority of the PLFA at this site (~50%) were C_{20} polyunsaturated FAMEs that are generally associated with phototrophic organisms such as diatoms and other algae (Volkman et al., 1989; Boschker et al., 2005). The next most abundant PLFA were the ubiquitous $C_{16:0}$ and $C_{18:0}$ (18.9% and 15.2%, respectively). These PLFA combined made up 85 mol% of the total FAMEs observed in the sample. The remainder of the PLFA were primarily $C_{16:1}$, $C_{18:1}$ and $C_{18:2}$, $C_{17:0}$ and $C_{20:0}$ and these comprised 15% of the total PLFA. The PLFA distribution in this sample is unusual, potentially due to the impact of the presence of petroleum contamination on the microbial community. Field observations indicated the recent colonization of the contaminated zone by a reddish epilithic community at the time of sampling. The monounsaturated PLFA observed in this sample are produced by both algal and bacterial organisms. In the absence of any more specific bacterial indicators, such as *iso*- or *anteiso*-branched PLFA, these PLFA are the only indicator of a bacterial heterotrophic component to this microbial community, suggesting that heterotrophic processes play a minor role in this community.

3.3. $\Delta^{14}C$ results

Four groups of microbial PLFA were chosen for ^{14}C analysis based on their potential carbon sources and abundance. The C_{20} polyunsaturated PLFA were collected as one sample representing the phototrophic organisms that make up the majority of this microbial community. The ubiquitous $C_{16:0}$ and $C_{18:0}$ PLFA were present in sufficient propor-

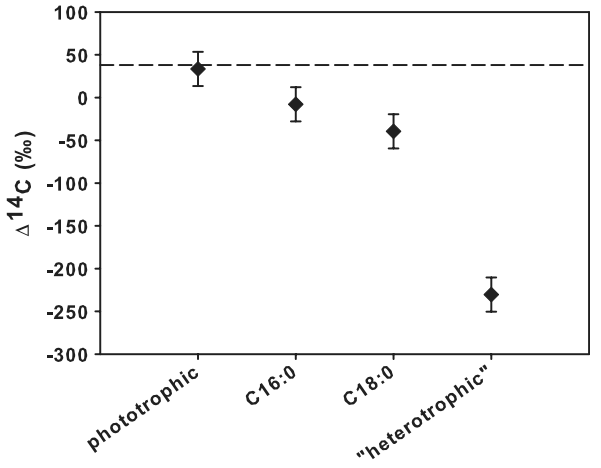

Fig. 4. $\Delta^{14}C$ PLFA compared to Buzzards Bay DIC showing the variation in $\Delta^{14}C$ between the different components of the microbial community. The phototrophic C_{20} unsaturated PLFA have a $\Delta^{14}C$ value that matches that of Buzzards Bay DIC. The pooled PLFA from the sample show a 260‰ depletion in $\Delta^{14}C$ due to incorporation of ^{14}C-depleted petroleum hydrocarbons. The ubiquitous $C_{16:0}$ and $C_{18:0}$ PLFA show varying extents of incorporation of the ^{14}C depleted carbon consistent with variation in the relative proportions of these PLFA produced by the heterotrophic and autotrophic components of the community.

tions to be collected individually. The remaining PLFA present in significant quantities were combined into a pooled sample of $C_{16:1}$, $C_{17:0}$, C_{18} unsaturates (several $C_{18:1}$ and $C_{18:2}$ isomers) and $C_{20:0}$ PLFA representing the remainder of the microbial community. As stated previously, these PLFA were likely contributed to by the primary phototrophic component of the community as well as by a minor heterotrophic component, however, due to limitations of chromatographic resolution and sample size, these PLFA were pooled together to represent the heterotrophic component of the microbial community and denoted "heterotrophic".

The $\Delta^{14}C$ contents of these four samples are shown in Fig. 4. The C_{20} polyunsaturated (algal) PLFA had a $\Delta^{14}C$ value of 33‰ that closely agrees with the $\Delta^{14}C$ of the dissolved inorganic carbon (DIC) of Buzzards Bay ($\Delta^{14}C = 38 \pm 14$‰, Fig. 4) in March 2003 (McNichol pers. comm.). This shows direct incorporation of Buzzards Bay DIC by algae, as would be expected during phototrophic activities. In contrast, the pooled "heterotrophic" PLFA sample has $\Delta^{14}C$ value of −230‰, depleted in $\Delta^{14}C$ by 263‰ relative to the C_{20} unsaturated PLFA from the same microbial community. This severe depletion could only be caused by heterotrophic uptake of a carbon source severely depleted in ^{14}C, such as the petroleum ($\Delta^{14}C = -1000$‰). The $\Delta^{14}C$ of the ubiquitous PLFA $C_{16:0}$ and $C_{18:0}$ were −8‰ and −39‰, respectively, consistent with a smaller, varying proportion of ^{14}C depleted carbon from the heterotrophic component of the community mixing with the modern phototrophic component. Were the depletion observed in the pooled sample due to a sampling artifact, these samples would not be expected to show any variation in $\Delta^{14}C$ with respect to the C_{20} polyunsaturated fatty acid sample.

The $\delta^{13}C$ values of the microbial PLFA (Table 1) are too ambiguous to interpret definitively. There is variation in $\delta^{13}C$, including some indication of potential isotopic depletion of some PLFA that may be a result of incorporation of isotopically depleted petroleum carbon ($\delta^{13}C = -26$‰). However, this variation is within the ranges that might be observed due to biosynthetic fractionations and thus cannot be considered as definitive evidence of petroleum carbon metabolism.

3.4. Implications for intrinsic biodegradation

The observed ^{14}C depletion in the pooled "heterotrophic" PLFA sample correlates with the increased rates of biodegradation of petroleum hydrocarbons indicated by the GC × GC analysis. This is strong evidence that the petroleum hydrocarbons are the source of this ^{14}C-depleted carbon and therefore represents direct, *in situ* evidence of the occurrence of biodegradation by the heterotrophic component of this microbial community. The other prevalent potential carbon source for the heterotrophic component of the community is respiration of detrital organics from the microbial community itself ($\Delta^{14}C = 33$‰) which could not produce such isotopically depleted PLFA. Dissolved organic carbon (DOC) from Buzzards Bay is also a potential carbon source that would be expected to be ^{14}C-depleted (Raymond and Bauer, 2001). Unfortunately, a recent $\Delta^{14}C$ analysis for Buzzards Bay DOC was not available, but the significance of DOC as a carbon source to this community adhered to the rocks in the inter-tidal zone is likely much less than either the petroleum or detrital sources.

Using the phototrophic PLFA ($\Delta^{14}C = 33$‰) and the petroleum ($\Delta^{14}C = -1000$‰) end-members, isotopic mass balance *via* Eq. (1) can be used to estimate the relative contribution of petroleum carbon to the pooled "heterotrophic" sample to be 26%.

$$\Delta^{14}C_{\text{pooled bacterial sample}} = f(\Delta^{14}C_{\text{petroleum}}) + (1-f)(\Delta^{14}C_{\text{algal}}) \quad (1)$$

This estimate represents a minimum petroleum contribution as any autotrophic PLFA present in the pooled "heterotrophic" sample ($\Delta^{14}C = -230‰$) would require an even greater contribution of petroleum carbon to the heterotrophic component (i.e., a 10% phototrophic contribution to the pooled sample implies the $\Delta^{14}C$ of the heterotrophic component must be $\Delta^{14}C = -259‰$, a 70% phototrophic contribution implies that the $\Delta^{14}C$ of heterotrophic component is $-845‰$). Similar isotopic mass balances indicate that 4% and 7% of the carbon in of the $C_{16:0}$ and $C_{18:0}$ PLFA, respectively, were generated from petroleum carbon, indicating that the heterotrophic component of the community is producing relatively more $C_{18:0}$ than the phototrophic component of the community.

These results imply several things about the intertidal community at Nyes Neck. The uptake of ^{14}C-depleted carbon confirms that intrinsic biodegradation by the heterotrophic component of the microbial community is contributing to the accelerated losses of petroleum hydrocarbons observed in the first month after the *Bouchard 120* oil spill. The agreement between polyunsaturated PLFA $\Delta^{14}C$ and $\Delta^{14}C$ DIC confirms phototrophic utilization of Buzzards Bay DIC. This also implies that no significant transfer of DIC is occurring from the heterotrophic metabolism of petroleum to the algal phototrophic system. Either heterotrophic metabolism is producing DIC at rates too low to affect the DIC pool utilized by the autotrophs, or this metabolism is occurring internal to the cell and this DIC is being utilized by the heterotrophs for biosynthesis directly.

4. Summary

In this study, molecular $\Delta^{14}C$ analysis of biomarker compounds has directly demonstrated, *in situ*, the carbon sources and metabolic activities of different components of a microbial community. This has confirmed that the observed accelerated losses of *n*-alkanes were related to intrinsic biodegradation by the heterotrophic, likely bacterial, component of the *in situ* microbial community and not by non-degradative processes such as evaporation and water washing. Such understanding of the relative role of components of a microbial community is essential to accurately understanding the persistence of, and therefore the risk posed by, organic contaminants in the environment.

Further, application of this approach requires only that the $\Delta^{14}C$ potential carbon sources of a microbial community be distinct and constrained. This difference is optimized when the potential carbon sources and modern and geologically old carbon, such as in the case of petroleum or petroleum based synthetic organics in the modern environment. But significant $\Delta^{14}C$ disparities can also be expected to be observed in other geologic systems that are of primary interest to research into microbial carbon sources and cycling such as hydrogen-utilizing microbial communities observed in the deep subsurface (Chappelle et al., 2002), anaerobic communities degrading petroleum hydrocarbons (Aitken et al., 2004) and endolithic communities (Walker et al., 2005).

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